

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 November 2002 (14.11.2002)

PCT

(10) International Publication Number  
WO 02/090531 A2

(51) International Patent Classification<sup>7</sup>: C12N 9/78, (74) Agent: VAN WEZENBEEK, P., M., G., F.; P.O. Box 20,  
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(21) International Application Number: PCT/EP02/04552

(81) Designated States (national): AE, AG, AL, AU, BA, BB,  
BG, BR, BZ, CA, CN, CO, CR, CU, CZ, DM, DZ, EC, EE,  
GD, GE, HR, HU, ID, IL, IN, IS, JP, KE, KP, KR, LC, LK,  
LR, LT, LV, MA, MG, MK, MN, MX, MZ, NO, NZ, PH,  
PL, RO, RU, SG, SI, SK, TT, UA, US, UZ, VN, YU, ZA.

(22) International Filing Date: 25 April 2002 (25.04.2002)

English

(25) Filing Language: English

(26) Publication Language: English

English

(30) Priority Data:  
01201601.0 3 May 2001 (03.05.2001) EP

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
(BF, BJ, CR, CG, CI, CM, GA, GN, GQ, GW, ML, MR,  
NE, SN, TD, TG).

**Published:**

— without international search report and to be republished  
upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 02/090531 A2

(54) Title: PEPTIDYLARGININE DEMINASE 6

(57) Abstract: A nucleotide acid sequence is provided encoding a peptidylarginine deiminase 6. The gene is found to be expressed in gonads only and may be used as target for male and female contraception. Its encoded protein can be used to screen for small molecular weight modulators of the enzyme activity.

## Peptidylarginine deiminase 6

The current invention relates to polynucleotides encoding peptidylarginine deiminase 6, cells transfected with these polynucleotides, proteins produced by these cells as well 5 as to a method to produce these proteins and its modulators.

Peptidylarginine deiminases (PADs) are a family of post-translational modification enzymes which convert peptidylarginine into citrulline in a  $\text{Ca}^{2+}$ -dependent manner. Enzymatic deimination *in vitro* changes the functional properties of various proteins 10 and alters their secondary and tertiary structures.

Sofar, five isoforms of PAD have been identified showing a broad tissue distribution. Mouse PAD1 is detected in the epidermis and uterus (Rus'd, A.A. *et al.* 1990, Eur. J. Biochem. 259, 660-669); murine PAD2 is widely expressed in various tissues such as brain, pituitary, spinal cord, salivary gland, pancreas, skeletal muscle, uterus, spleen, 15 stomach and thymus (Takahara, H. *et al.* 1989, J. Biol. Chem. 264, 13361-13368); murine PAD3 is expressed in epidermis and hair follicles (Terakawa, H. *et al.* 1991, J. Biochem. (Tokyo) 110, 661-666); PAD4 (rat) is an ubiquitous enzyme being expressed in the pancreas, spleen, ovary, liver, lung, stomach, kidney, uterus, dermis, brain, heart and epidermis (Yamakoshi, A. *et al.* 1998, Biochim. Biophys. Acta 1386, 227-232); 20 human PAD5 finally has been isolated as a new family member from a myeloid leukemia cell line, but its tissue distribution has not been further determined (Nakashima, K. *et al.* 1999, J. Biol. Chem. 274, 27786-27792).

Little is known about the physiological functions of PAD. In brain, myelin basic protein is a natural substrate and therefore, PAD plays an important part in the central 25 nervous system. Moreover, when disregulated PAD plays a role in the aetiology of multiple sclerosis (Mastronardi, F.G. *et al.* 1996, Clin. Invest. 97, 349-358). PAD in the epidermis seems to be involved in the terminal processing of filaggrin, which indirectly is important for the maintenance of moisture in the upper stratum corneum (Senshu, T. *et al.* 1996, Biochem. Biophys. Res. Commun. 225, 712-719). Again, disregulation of 30 this PAD may play a role in the aetiology of rheumatoid arthritis (Girbal-Neuhauser, E. *et al.* 1999, J. Immunol. 162, 585-594). In hair follicles finally the solubility of trichohyalin seems to be influenced by PAD; the function of this remains to be determined (Rogers, G.E. *et al.* 1997, J. Invest. Dermatol. 108, 700-707).

We now have found a novel PAD, which is called PAD6. The transcript has been found in mouse oocytes. Its human homologue is also described herein. The protein was found to be expressed exclusively in oocytes/ovary and testes.

- 5    Genes that are expressed specifically in male and/or female gametes may provide novel molecular targets for male and female contraception. For testis, large numbers of gene sequences expressed uniquely in germ cells have been described (Pawlak, A. *et al.* 1995 *Genomics* **26**, 151-1588; Wolgemuth D.J. and Watrin F. 1991 *Mamm Genome* **1**, 283-817). In contrast, only a few genes specifically expressed in oocytes thus far have
- 10    been identified. The majority of gamete specific gene sequences identified are likely to have an essential function due to their specific expression in gametes. The latter is confirmed by studies using knockout animals indicating that gene inactivation of testis and oocyte specific genes generally results in male and/or female infertility but does not result in additional pathology in other organs and tissues (Dong, J. *et al.* 1996 *Nature* **383**, 531-535; Nantel, F. *et al.* 1996, *Nature* **380**, 159-162). These data provide further evidence for the specific and essential role of these genes during gametogenesis. This underlines the importance of tissue specificity as selection criterion for molecular targets for fertility regulation.
- 15
- 20    It will be clear that there is a great need for the elucidation of genes involved in fertility regulation in order to unravel the various roles these genes may play in infertility. A better knowledge of the genes involved in different stages of female and male fertility e.g. in gametogenesis and their activity and expression regulation might help to create a better insight in infertility disorders. This could eventually lead to the identification of activity modulators to be used in either *in vivo* or *in vitro* therapeutic protocols.
- 25

The present invention provides for such a gene. More specific, the present invention provides for a polynucleotide sequence encoding peptidylarginine deiminase 6 (PAD6). Preferably the polynucleotide is of mammalian origin, preferably mouse, more preferably human. The RNA is expressed exclusively in reproductive organs.

- 30    The most preferred polynucleotide sequences are those encoding SEQ ID NO: 1 or SEQ ID NO:3.

The invention also includes the entire mouse mRNA sequence as indicated in SEQ ID NO:2 and more specifically the open reading frame corresponding to nucleotide sequence 6-2051 of SEQ ID NO:2. This sequence encodes a protein of 682 amino acids (SEQ ID NO:1). In addition the invention includes the entire human mRNA sequence

as indicated in SEQ ID NO:4 and the open reading frame corresponding to nucleotide sequence 20-2077 of SEQ ID NO:4. This sequence encodes a protein of 686 amino acids (SEQ ID NO:3). To accommodate codon variability, the invention also includes polynucleotide sequences coding for the same amino acid sequences as the sequences disclosed herein. The sequence information as provided herein should not be so narrowly construed as to require exclusion of erroneously identified bases. The specific sequence disclosed herein can readily be used to isolate the complete genes of several other species or allelic variants. The sequence can e.g. be used to prepare probes or as a source to prepare synthetic oligonucleotides to be used as primers in DNA 10 amplification reactions allowing the isolation and identification of the complete variant genes. In particular, polynucleotides hybridizing under stringent washing conditions with a probe prepared with PCR under standard conditions using SEQ ID NO:14 and SEQ ID NO:15 with cDNA from mammalian origin as a template, preferably human or mouse, are part of the invention. Such a probe (and its complementary sequence) is 15 identified e.g. by the nucleotides 464-1052 of SEQ ID NO:4.

The complete genetic sequence can be used in the preparation of vector molecules for expression of the protein in suitable host cells.

Thus, in one aspect, the present invention provides for isolated polynucleotides 20 encoding the novel PAD6 protein. Preferably the PAD6 is of human origin, but also orthologs form part of the invention.

The DNA according to the invention may be obtained from cDNA. The tissues preferably are from mammalian origin, more preferably from human origin. Preferably ribonucleic acids are isolated from oocytes or testes. Alternatively, the coding sequence 25 might be genomic DNA, or prepared using DNA synthesis techniques. The polynucleotide may also be in the form of RNA. If the polynucleotide is DNA, it may be in single stranded or double stranded form. The single strand might be the coding strand or the non-coding (anti-sense) strand.

30 The DNA according to the invention will be very useful for *in vivo* or *in vitro* expression of the novel protein according to the invention in sufficient quantities and in substantially pure form.

The present invention further relates to polynucleotides having slight variations or having polymorphic sites. Polynucleotides having slight variations may encode variant

polypeptides which retain the same biological function or activity as the natural, mature protein. Polymorphic sites are useful for diagnostic purposes.

In another aspect, the invention provides for a method to isolate a polynucleotide comprising the steps of: a) hybridizing a polynucleotide according to the present invention, or its complement, under stringent conditions against nucleic acids being (genomic) DNA RNA, or cDNA isolated preferably from tissues which highly express the polynucleotide of interest and b) isolating said nucleic acids by methods known to a skilled person in the art. The tissues preferably are from human origin. Preferably ribonucleic acids are isolated from oocytes, ovaria or testes. The hybridization conditions are preferably highly stringent.

According to the present invention the term "stringent" means washing conditions of 1 x SSC, 0.1% SDS at a temperature of 65 °C; highly stringent conditions refer to a reduction in SSC towards 0.3 x SSC, more preferably to 0.1 x SSC. Preferably the first two washings are subsequently carried out twice each during 15-30 minutes. If there is a need to wash under highly stringent conditions an additional wash with 0.1 x SSC is performed once during 15 minutes. Hybridization can be performed e.g. overnight in 0,5M phosphate buffer pH7.5/7% SDS at 65 °C.

As an alternative the method to isolate the gene might comprise gene amplification methodology using primers derived from the nucleic acid according to the invention. Complete cDNAs might also be obtained by combining clones obtained by e.g. hybridization with e.g. RACE cDNA clones.

Also portions of the coding sequences coding for a functional polypeptide are part of the invention as well as allelic and species variations thereof. Sometimes, a gene is expressed in a certain tissue as a splicing variant, resulting in an altered 5' or 3' mRNA or the inclusion or exclusion of one or more exon sequences. These sequences as well as the proteins encoded by these sequences all are expected to perform the same or similar functions and form also part of the invention.

The invention also provides for peptidylarginine deiminase 6 (PAD6). Preferably the protein has a mammalian amino acid sequence, more preferably a human sequence. Most preferred are the sequences as described in SEQ ID NOs: 1 or 3. Expression can be obtained by introduction of vector molecules comprising a polynucleotide encoding PAD6 into suitable host cells. The cells can be cultured and the protein can be isolated using methods known to the person skilled in the art.

- 5 -

In still another aspect of the invention there are provided functional equivalents that is polypeptides encoding PAD6 activities and comprising essentially the same SEQ ID NO:1 or 3 sequence or parts thereof having variations of the sequence while still maintaining functional characteristics.

5 The variations that can occur in a sequence may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions that are expected not to essentially alter biological and immunological activities, have been described. Amino acid replacements between related amino acids or replacements  
10 which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Based on this information Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science, 1985 227, 1435-1441) and determining the functional similarity  
15 between homologous polypeptides. It will be clear that also polynucleotides coding for such variants are part of the invention.

Thus, in another aspect of the invention there are provided polypeptides comprising SEQ ID NO:1 or SEQ ID NO:3 or but also polypeptides with a similarity of 80%, preferably 90%, more preferably 95%.

20 As used herein the term similarity is as defined in NCBI-BLAST 2.0.10 [Aug-26-1999] (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25, 3389-3402). The program is used to search for sequence alignments using default  
25 settings. For amino acid alignments the BLOSUM62 matrix is used as a default and the similarity is indicated as the number of positives. No filtering of low compositional complexity is included.

Also portions of such polypeptides still capable of conferring biological effects are included. Especially portions which still are capable of converting arginine to citrulline  
30 form part of the invention. Such proteins or functional parts thereof may be functional per se, e.g. in solubilized form or they may be linked to other polypeptides (e.g. to direct it to specific subcellular compartments, to increase its stability or to facilitate its purification), either by known biotechnological ways or by chemical synthesis, to obtain chimeric proteins.

It will be clear that also polynucleotides encoding such variant polypeptides are included in the invention.

A wide variety of host cell and cloning vehicle combinations may be usefully 5 employed in cloning the nucleic acid sequence coding for the polypeptide according to the invention.

Suitable expression vectors are for example bacterial or yeast plasmids, wide host range plasmids and vectors derived from combinations of plasmid and phage or virus DNA. Vectors derived from chromosomal DNA are also included. Furthermore an 10 origin of replication and/or a dominant selection marker can be present in the vector according to the invention. The vectors according to the invention are suitable for transforming a host cell.

Vehicles for use in expression of the protein or parts thereof of the present invention will further comprise control sequences operably linked to the nucleic acid sequence 15 coding for the protein. Such control sequences generally comprise a promoter sequence and sequences, which regulate and/or enhance expression levels. Of course control and other sequences can vary depending on the host cell selected.

Recombinant expression vectors comprising the DNA of the invention as well as cells transfected with said DNA or said expression vector, either transiently or stable, also 20 form part of the present invention.

Suitable host cells according to the invention are bacterial host cells, yeast and other fungi, plant or animal host such as Chinese Hamster Ovary cells, monkey cells, or human cells. Thus, a host cell which comprises the DNA or expression vector according to the invention is also within the scope of the invention. The engineered 25 host cells can be cultured in conventional nutrient media which can be modified e.g. for appropriate selection, amplification or induction of transcription. The culture conditions such as temperature, pH, nutrients etc. are well known to those ordinary skilled in the art.

30 The techniques for the preparation of the DNA or the vector according to the invention as well as the transformation or transfection of a host cell with said DNA or vector are standard and well known in the art, see for instance Sambrook et al., Molecular Cloning: A laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

The protein according to the invention can be recovered and purified from recombinant cell cultures by common biochemical purification methods (as described in *Guide to Protein purification*. Edited by Murray P. Deutscher. (1990) *Methods in Enzymology*.

5 Vol 182. Academic Press, inc. San Diego CA 92101. Harcourt Brace Jovanovich, Publishers). including ammonium sulfate precipitation, extraction, chromatography such as hydrophobic interaction chromatography, cation or anion exchange chromatography or affinity chromatography and high performance liquid chromatography. If necessary, also protein refolding steps can be included.

10 Alternatively the protein can be expressed and purified as a fusion protein containing ("tags") which can be used for affinity purification.

Regulation of the activity of the protein according to the invention is useful *in vivo* for the control of follicular recruitment, but also of growth and maturation of oocytes and/or follicles. Inhibition of these processes *in vivo* can be used to delay (premature) menopause and/or as a contraceptive. In addition, the protein can be employed for *in vitro* maturation and growth of follicles e.g. from frozen ovarian tissue.

20 PAD gene products according to the present invention can be used for the *in vivo* or *in vitro* identification of novel substrates or analogs thereof. For this purpose e.g. peptidyl arginine deiminase assay studies can be performed with cells transformed with DNA according to the invention or an expression vector comprising DNA according to the invention, said cells expressing the PAD6 gene products according to the invention. Alternatively also the PAD6 protein itself or the substrate-binding domains thereof can 25 be used in an assay for the identification of functional substrates or analogs.

Methods to determine peptidyl arginine deiminase activity of expressed gene products in *in vitro* and *in vivo* assays to determine biological activity of gene products are well known. See e.g. Lamensa, F.E.W. and Moscarello, M.A. 1993 *J. Neurochem.* 61, 987-996. In this assay arginine in  $\alpha$ -Benzoyl-Larginine ethyl ester (BAEE) is converted 30 in citrulline which can easily be measured after precipitation with perchloric acid.

Another example of determining the enzymatic activity of PAD6 makes use of the inactivation of a protein e.g. Soybean Trypsin Inhibitor (STI) (Takahara, H. *et al.* 1985, *J. Biol. Chem.* 260, 8378-8383. When an essential arginine in STI is converted into citrulline it is no longer able to inhibit the proteolytic activity of trypsin. This can be 35 used as the basis for a two-step assay for the determination of PAD activity. The assay

consists of two steps. In the first reaction PAD converts the arginine (position 63) in STI into a citrulline inactivating the STI. In the second reaction trypsin and a fluorescent substrate are added and trypsin activity is measured.

5 Alternatively modulation of the PAD6 activity can also be obtained by downregulation of the expression level of the protein e.g. by using anti-sense nucleic acids through triple-helix formation (Cooney *et al.*, 1988, *Science* 241, 456-459) or by binding to the mRNA, or by influencing mRNA stability or protein interactions by small molecules. This in itself could also lead to regulation of fertility i.e. contraception or treatment of  
10 infertility.

Thus, the present invention provides for a method for identifying compounds that affect the enzymatic function of the protein according to the invention. The method comprises the steps of

15 a) contacting the PAD6 protein with an arginine containing substrate  
b) contacting said mixture with a test compound  
c) measuring the arginine to citrulline conversion and  
d) comparing said conversion with peptidylarginine deiminase activity in the absence of a test compound.  
20 The arginine to citrulline conversion can easily be measured e.g. by analytical methods like HPLC, altered proteolytic sensitivity of the peptide, change in activity properties of the peptide or specific antibody recognition. As a substrate peptides or proteins comprising arginine can be used, but also synthetic compounds such as  $\alpha$ -N-benzoyl-L-arginine ethyl ester can be used. However, the amino and carboxyl groups have to be  
25 substituted or have to be in a peptide bonded form.

Alternatively, the present invention provides for a method to identify compounds that modulate the PAD6 mRNA stability or the PAD6 expression levels.

The present invention thus provides for a quick and economic method to screen for therapeutic agents for fertility control related to the activity of PAD6. The method  
30 according to the invention furthermore provides for the selection of selective therapeutic agents discriminating between different peptidylarginine deiminases thus leading to a more effective therapeutic agent and/or diminishing of side effects. The method is especially suited to be used for the high throughput screening of numerous potential target compounds.

Compounds which modulate the peptidylarginine deiminase 6 function may be employed in therapeutic treatments by modulating the PAD of the present invention.

The invention also provides for a method for the formulation of a pharmaceutical composition comprising mixing the modulator compounds identified with a pharmaceutically acceptable carrier.

5 Pharmaceutical acceptable carriers are well known to those skilled in the art and include, for example, sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil and water.

Furthermore the pharmaceutical composition may comprise one or more stabilizers 10 such as, for example, carbohydrates including sorbitol, mannitol, starch, sucrosedextrin and glucose, proteins such as albumin or casein, and buffers like alkaline phosphates. Methods for making preparations and intravenous admixtures are disclosed in Remington's Pharmaceutical Sciences, pp. 1463-1497 (16th ed. 1980, Mack Publ. Co of Easton, Pa, USA).

15 Thus, the modulator compounds identified by using the peptidylarginine deiminase according to the invention are useful in the preparation of a pharmaceutical. The pharmaceutical is to be used for control of fertility disorders.

20 The following examples are illustrative for the invention and should in no way be interpreted as limiting the scope of the invention.

### Legends to the figures

#### Figure 1

RT-PCR analysis (30 cycles) of mouse PAD6 expression in various mouse tissues 25 (upper panel). In the lower panel GAPDH controls in the absence and presence of RT are shown.

#### Figure 2

ISH (In Situ Hybridization) analysis using clone 1B11 as a probe on ovaries from young (7 days) and adult mice.

30 S= secondary follicle      A=antral follicle

**Figure 3**

RT-PCR analysis (30 cycles) of human PAD6 expression in various human tissues (upper panel). In the lower panel GAPDH controls in the absence and presence of RT are shown.

**5 Figure 4**

Human multiple tissue northern blots (Clontech) hybridised with hPAD6 probe.

**Figure 5**

Fluorescence measurement to determine PAD activity. STI (0.17  $\mu$ g) was pre-incubated in the absence (filled square) or presence of 0.5  $\mu$ g GST-PAD6 (filled triangle), 1.0  $\mu$ g GST-PAD6 (open triangle), or 0.1  $\mu$ g rabbit muscle PAD (open square; Sigma cat. No P1584) respectively. Subsequently,  $\text{Na-benzoyl-L-Arginine-7-amido-4-methylcoumarin}$  (100  $\mu$ M; Sigma cat. No. B7260) and trypsin (0.25  $\mu$ g) were added, and fluorescence determined.

**Examples****15 Example 1 Preparation of mouse cDNA clones***Generation of oocyte cDNA library.*

Total RNA was isolated from 2172 denuded mouse oocytes, treated *in vitro* for 15h with 50  $\mu$ M FF-MAS, according to the RNAzol B<sup>TM</sup> RNA isolation protocol (Campro scientific). RNAzol B was added directly to the frozen cell pellets containing approximately 100 oocytes each. Homogenates were pooled and extracted with 0.1 volume of chloroform, shaken for 15 seconds and incubated on ice for 10 minutes. After centrifugation for 15 minutes at 14000 rpm at 4°C the aqueous phase was collected. Total RNA was precipitated by adding an equal volume of isopropanol followed by o/n incubation at 4°C. RNA was centrifuged for 45 minutes at 14000 rpm at 4°C, the pellet was washed once with 700  $\mu$ l of 70% ethanol followed by centrifugation at 14000 rpm at 4°C for 30 minutes. The air-dried pellet was finally resuspended in 7.5  $\mu$ l Rnase free water (Ambion). The total amount of RNA isolated using this procedure was determined using the Ribogreen<sup>TM</sup> RNA quantitation kit (Molecular Probes).

30 For cDNA synthesis, the SMART<sup>TM</sup> PCR library construction kit (Clontech) was used. The following modifications were introduced. An oligodT(18) primer with EcoRI restriction site (Pharmacia) was annealed to the 3' end of the mRNA and the

SMART™ oligo extended with an EcoRI restriction site was annealed to the 5' end of the mRNA. The first strand cDNA synthesis reaction was in a reaction buffer containing 50 mM Tris (pH 8.3), 75 mM KCl, 6 mM MgCl<sub>2</sub>, 2 mM DTT, 1 mM dNTP mix and 200 units Superscript II RNase H Reverse transcriptase (Gibco BRL) for 1 hour at 42°C. Subsequently first strand cDNA was amplified by PCR using a Perkin Elmer thermocycler (9600). The PCR was performed in a total volume of 100 µl reaction buffer containing 1x Klen Taq PCR buffer (Clontech), 0.2 mM dNTP mix (Clontech), 0.2 mM 5' EcoRI-SMART primer, 0.2 mM NotI-EcoRI-dT(18) primer (Pharmacia) and 1x Advantage Klen Taq Polymerase Mix (Clontech) starting with 1 minute denaturation at 95°C followed by 28 cycles of 15 seconds at 95°C and 5 minutes at 68°C.

After purification on a Qiaquick spin column (QIAGEN) the cDNA was digested with EcoRI (Pharmacia) at 37°C followed by heat inactivation at 70°C for 10 minutes. cDNA was purified twice using two subsequent Qiaquick spin columns and finally resuspended in 50 µl 10 mM Tris-Cl (pH 8.5). DNA concentration was determined by measuring adsorbance at 260 nm using a Genequant spectrophotometer.

#### *Size fractionation of cDNA*

cDNA was size fractionated using agarose gel electrophoresis and extracted from the gel matrix using the QiaexII Agarose Gel Extraction Kit (Qiagen). DNA was eluted in 20 µl H<sub>2</sub>O, purified on a Qiaquick spin column (Qiagen) and eluted in 50 µl H<sub>2</sub>O. The samples were precipitated by adding 0.1 volume of 3M Sodium Acetate, 10 µg of glycogen and 2.5 volumes of ethanol (96% v/v) followed by 1h incubation at -20°C. The size fractionated cDNA was collected by centrifugation at 14.000 rpm for 20 minutes at 4°C. The DNA pellet was washed with 70% ethanol and air dried before it was dissolved in MQ. DNA concentration was determined using the PicoGreen™ dsDNA Quantitation Kit (Molecular Probes).

After EcoRI digestion, 200 ng oocyte cDNA was ligated into 500 ng of predigested and dephosphorylated λGT11 phage arms in a buffer containing 50mM Tris-Cl pH 7.8, 10 mM MgCl<sub>2</sub>, 10 mM dithiotreitol, 1 mM ATP, and 750 units /ml T4 ligase (Pharmacia). The reactions were incubated o/n at 16°C. The complete ligation reaction was finally packaged into a Max Plax™ packaging extract (Epicentre) as described in the product information sheet.

## Example 2 Isolation and characterization of mouse PAD6

### *PCR amplification of phage clones*

Single plaques were incubated for at least one hour in 100  $\mu$ l  $\lambda$  phage buffer (10 mM Tris-HCl pH 8.3, 100 mM NaCl<sub>2</sub> and 10 mM MgCl<sub>2</sub>). From each eluted plaque 2.5  $\mu$ l

- 5 was PCR-amplified using  $\lambda$ GT11 primers (SEQ ID NO:5 and SEQ ID NO:6). PCR reactions were performed on the PE9700 (9600 mode, Perkin Elmer), one cycle of 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 55°C and 3 min at 72°C, followed by one cycle of 5 min at 72°C. PCR products were analyzed by agarose gel electrophoresis and selected on size, purity and concentration. Only single bands of 500
- 10 bp or more were selected for sequencing.

### *DNA Sequence analysis*

750 clones from the mouse oocyte cDNA library were analyzed by DNA sequencing after insert amplification by PCR. Sequence analysis was performed using the Big Dye

- 15 DNA sequencing ready reaction protocol (Perkin Elmer) and samples were analyzed on the ABI377 automatic DNA sequencer (Perkin Elmer). Sequences were blasted against several databases a.o.: gb111rod, genpept, EMrodESTs59 and EMhumanESTs59 databases using BLASTN or TBLASTN in an automated procedure and annotated on basis of homology to gene(s) with known functions.

20

### *Identification and characterization of PAD6.*

One of the sequences obtained shows strong homology with peptidyl arginine deiminase III. Based on homology searches it has been established that this clone, 1B11, encodes a novel peptidyl argine deiminase that has been termed PAD6.

25

The 5'-end of mouse PAD6 cDNA could be amplified from a mouse ovary cDNA library. The cDNA of this library had been cloned directionally into NotI-Sall sites (5'-3') of the pSPORT vector (Life Technologies). This vector contains the M13 forward and SP6 promotor sequences 5' from the NotI site which have been used in the 5'

- 30 RACE PCR in combination with two PAD6 specific reverse primers. The first PCR was performed with the M13F primer (SEQ ID NO:7) and the gene specific reverse primer (SEQ ID NO:8). This PCR product was diluted fifty times and one microliter of this dilution was used as template in the nested PCR with the SP6 primer (SEQ ID NO:9) and the nested gene specific reverse primer (SEQ ID NO:10). Both PCR

reactions were performed in a total volume of 50  $\mu$ l reaction buffer containing 1X Klen Taq PCR buffer (Clontech), 0.2 mM dNTP mix (Clontech) and 1X Advantage Klen Taq Polymerase Mix (Clontech) starting with 5 minutes of denaturation at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 3 minutes at 72°C 5 with an final extension of 5 minutes at 72°C.

Bands in the nested PCR products were cloned in the TA Topo PCR2.1 vector (Invitrogen) following the product information sheet and sequenced. It was found that a 1800 bp 5' RACE fragment completed the mouse PAD6 clone. The sequence of the full-length mouse cDNA is given in SEQ ID NO:2.

10 Based on DNA sequence information obtained, gene specific PCR primer sets were designed and used in RT-PCR experiments to confirm the tissue-specific expression profile. The data obtained (Figure 1) confirm the oocyte/ovary- (and testis-)specific expression for mouse PAD6. (SEQ ID NO:8 and SEQ ID NO:13 were used as primers).

15

#### *In situ hybridization (ISH)*

To further study the expression of PAD6 in the gonads, *in situ* hybridization (ISH) was performed on sections of mouse ovary and testis.

20 Ovaries of day 7 and adult mice were fixed in 4% buffered formalin for 24 hours at room temperature. The tissues were embedded in paraffin. Paraffin sections (5  $\mu$ m) were cut, mounted on Superfrost plus microscope slides, and allowed to dry overnight at 37 °C. The slides were baked at 60 °C for two hours.

25 Tissue sections were dewaxed in xylene en rehydrated in descending concentrations of ethanol. Slides were washed for 20 min shaking in 0.2M HCl, followed by two washes in DEPC (di-ethylpyrocarbonate) treated Milli Q. The sections were treated with proteinase K (1  $\mu$ g/ml) in digest buffer (100 mM Tris, 50 mM EDTA pH 8) for 30 min at 37 °C. Digestion was stopped in prechilled 0.2% glycine in PBS for 10 min shaking at room temperature (RT). The slides were acetylated for 5 min with 0.25 % acetic 30 anhydride in 0.1 M triethanolamine buffer, followed by two washes in DEPC treated Milli Q. Sections were prehybridised at hybridisation temperature in a humid chamber with prehybridisation mix, containing 52% formamide, 21 mM Tris, 1 mM EDTA, 0.33 M NaCl, 10% dextran sulphate, 1x Denhardt's solution, 100  $\mu$ g/ml salmon sperm DNA, 100  $\mu$ g/ml tRNA and 250  $\mu$ g/ml yeast total RNA. The slides were covered with

a glass coverslip. After two hours coverslips were replaced by coverslips holding 100  $\mu$ l probe hybridization mix, containing prehybridization mix with the following additions: 0.1 mM DTT, 0.1% sodium thiosulphate, 0.1% SDS and 200 ng/ml DIG-labeled probe.

5

DIG-labeled probes were generated by *in vitro* transcription from a linear DNA template, using DIG-dUTP and DNA-dependent RNA polymerases (SP6 and T7). The promoter site of each RNA polymerase was attached to gene specific sequences allowing the generation of a PCR fragment containing the SP6 promoter site at the 5' 10 and the T7 promoter at the 3' site. In general, probes from about 250-500 nucleotides were made located at the 5' end of SEQ ID NO:2. After *in vitro* transcription a small amount of the probe was analyzed on a 1.5% agarose gel to confirm successful *in vitro* transcription. Probe concentrations were estimated by spotting serial dilutions (including control DIG-*RNA* (100 ng/ $\mu$ l)) on a Hybond N<sup>+</sup> membrane followed by 15 anti-DIG alkaline phosphatase Fab' fragments (anti-DIG-AP) and NBT/BCIP colour substrate incubation.

The hybridization was carried out overnight (16 hours) in a humid chamber at 42 °C or 50 °C. Slides were then washed in 2x SSC, shaking for 15 min, followed by washes in 20 2x SSC, 1x SSC and 0.1x SSC for 15 min shaking at hybridization temperature. Sections were digested by Ribonuclease A (20  $\mu$ g/ml) in RNase buffer (0.6 M NaCl, 20 mM Tris, 10 mM EDTA) for 1 hour at 37 °C. After two washes (5 min shaking RT) in prechilled PBS and one wash in buffer 1 (100 mM maleic acid, 150 mM NaCl), the sections were incubated for 30 min with blocking solution (1 g/ml blocking reagent in 25 buffer 1). Then the sections were incubated with anti-DIG-AP, diluted 1:500 in blocking solution, for 1 hour at RT. After two washes in buffer 1 (15 min shaking RT), the slides were carefully wiped dry around the tissue and the sections were encircled with a DAKO-pen. The sections were covered with NBT/BCIP colour development 30 reagent and incubated in a humid chamber at RT. After two hours the sections were examined under a microscope. If no or only weak staining was observed the incubation was continued overnight at 4 °C and the next day at RT. Finally, the slides were rinsed in water and optionally counterstained with Mayer's hematoxyline 1:5 for three seconds. Slides were mounted in Kaisers glycerol gelatin.

As shown in Figure 2, PAD6 is expressed in the ovary exclusively in oocytes.

PAD6 mRNA has high expression levels in oocytes of primary, secondary and antral follicles, but is also expressed in oocytes from primordial follicles. Based on the data obtained sofar the expression level of PAD6 mRNA decreases in oocytes of antral follicles suggesting that the function of PAD6 is most likely required during early stages of oogenesis. Although RT-PCR data revealed testis expression of PAD6, no expression above background level of PAD6 mRNA was detected using ISH analysis suggesting low levels of expression of PAD6 in the testis.

10 **Example 3 Isolation and characterization of human PAD6**

A BLAST search using the full-length mouse PAD6 cDNA as a query against the EM63hsGeno(new) databases identified the human homologue of PAD6. This search only identified the C-terminal region of the coding sequence of human PAD6. To 15 extend the sequence in the 5' direction primers were designed and a 5' RACE PCR was performed on human ovary Marathon Ready cDNA (Clontech) using the Marathon Ready™ cDNA user manual. The first PCR was performed under the following conditions: a denaturation of 30 seconds at 94°C, 5 cycles of 5 seconds at 94°C and 3 minutes at 72°C, 5 cycles of 5 seconds at 94°C and 3 minutes at 70°C and 25 cycles of 20 5 seconds at 94°C and 3 minutes at 68°C. A 50-fold dilution of this first PCR product served as template in the second, nested PCR reaction using the same PCR conditions. An expected band of ≈650 bp was cloned in the TA Topo PCR2.1 vector (Invitrogen) and sequenced. This clone contained (by homology) the first 5' 500 base pairs of the coding sequence of human PAD6, thus completing the coding sequence of human 25 PAD6.

PCR primers were selected to amplify the full length human PAD6 cDNA human ovary RNA. For isolation of human PAD6 cDNA the primers SEQ ID NO:11 and SEQ ID NO:12 were used on Marathon Ready ovary cDNA (Clontech). PCR conditions were: denaturation for 5 minutes at 94°C followed by 5 cycles of 30 seconds at 94°C 30 and 3 minutes at 68°C, 28 cycles of 30 seconds at 94°C, 30 seconds at 62°C and 3 minutes at 72°C with a final extension of 7 minutes at 72°C.

The full length amplicates of three independent PCR reactions were cloned into the PCR2.1 Topo vector (Invitrogen) and sequenced to determine the consensus nucleotide sequences. Its sequence is shown in SEQ ID NO:4.

Gene specific PCR primer sets were designed (SEQ ID NO:14 and SEQ ID NO:15) and used in RT-PCR experiments to determine the expression profile of human PAD6. RT-PCR on RNA from human testis, uterus, kidney, thymus, liver, brain, heart, lung and spleen, revealed PAD6 expression only in testis (Figure 3).

5

Multiple Tissue Northern Blots (Clontech) of human tissues were hybridised with the PCR fragment of human PAD6 (approximately 590 bp; PCR product of primers SEQ ID No:14 and SEQ ID NO:15 extending from nucleotides 464-1052 in SEQ ID NO:4). Probes were labelled with [<sup>32</sup>P]dCTP and Ready to Go Labellings beads (AP Biotech) 10 according to the manufacturer's instruction using an incubation time of 60 minutes at 37°C. The non-incorporated dNTP's were removed on a spin column of Sephadex G50 in a 1ml syringe.

The blots were prehybridised in Express hybmix (Clontech) for at least one hour at 15 65°C. For hybridisation 4-8 x 10<sup>7</sup> cpm of the denatured probes were added to the prehybridisation mixture. The blots were hybridised at 65°C overnight and washed once with 2X SSC, 0.1% SDS at room temperature, twice with 1X SSC, 0.1% SDS at 65°C and once with 0.1X SSC, 0.1% SDS at 65°C. The hybridised blots were analysed with the STORM 840 Phosphor imager (Molecular Dynamics), scanned on 200 micron and printed with a range of 0-50 after exposure of three days to Kodak storage 20 phosphor screens GP (Molecular dynamics).

In Figure 4 a single band in ovary with an estimated length of about 3 kB can be seen showing up only in ovary. No signal could be detected in testis, most likely because the level of PAD6 expression in testis is too low to be detected on Northern blots. *In situ* hybridisation analysis corroborates these results: PAD6 expression could be detected in 25 all types of follicles of human and monkey ovaries and are in this respect similar to the *in situ* data in mouse. By *in situ* hybridisation no expression was detected in testis (data not shown).

#### Example 4 Expression of human PAD6 and determination of PAD6 activity

30 *Cloning*

Full-length human PAD6 was cloned into the bacterial expression vector pGEX4T1 (AP Biotech) using the Rapid DNA Ligation kit (Boehringer). The recombinant construct (pGEXhPAD6) was characterised by restriction enzyme digestion.

- 17 -

*E. coli* BL-21 cells transformed with pGEXhPAD6 were grown in 2 x YT medium at 25°C to a cell density of 1.0 at 650 nm. After addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside the culture was grown for an additional 5 hours at 25°C. The cells were centrifuged and resuspended in 0.1 vol of the original culture volume of 20 mM Tris-HCl, pH 7.6, 1 mM EDTA and lysed by sonication on ice. The sonicate was centrifuged at 15000 X g for 30 minutes at 4°C (Sorvall, SS34 rotor) and to the supernatant 1M NaCl, 0.1% of Triton X-100 and 50% glutathion-Sepharose 4B beads in PBS (Pharmacia Biotech, 1 ml to an equivalent of 250 ml initial culture) was added, followed by incubation at 4°C for 60 minutes with gentle agitation. The beads were then washed three times with 10 bed volumes of a buffer containing 20 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.1% Triton X-100 and 0.1 M NaCl at RT for 5 minutes with gentle agitation. The recombinant hPAD6-gst fusion protein was eluted from the beads in several steps with 10 to 100 mM reduced glutathione in 50 mM Tris-Cl pH 8.0, 0.1 M NaCl and 0.1% Triton X-100 at 4°C for 30 minutes with gentle agitation. The eluates were stored with 10% glycerol at -20°C for determination of enzymatic activity. The purity of the protein was estimated to be 90% based on SDS PAGE analysis.

*Determination of PAD6 enzyme activity.*

The activity of the PAD was determined by the formation of citrulline in Soybean Trypsin Inhibitor (STI) as a substrate. In contrast to the original STI, citrullinated STI is unable to inhibit trypsin activity. Therefore, an increased activity of trypsin, as detected with a fluorescent trypsin substrate, indicates PAD activity.

For PAD activity, the reaction mixture consisted of 100 mM HEPES (pH 7.5), 5 mM CaCl<sub>2</sub>, 2 mM DTT, 0.17 µg STI and an aliquot of the purified enzyme solution [either GST-PAD6 or the commercial available PAD (Sigma), derived from rabbit muscle] in a final volume of 20 µl. After incubation of the assay mixture for 30 minutes at 37 °C, 10 µl of the fluorescent substrate Na-benzoyl-L-Arginine-7-amido-4-methylcoumarin [400 µM in 100 mM HEPES (pH 7.5), 50mM EDTA] and 10 µl of trypsin solution [0.25 µg in 100 mM HEPES (pH 7.5)] were added subsequently. Fluorescence measurements (excitation 360 nm, emission 460 nm) were started directly in a Victor V at room temperature, and were continued for one hour.

PAD6 activity could be detected as can be seen in Figure 5.

## Claims

1. A polynucleotide comprising a nucleotide sequence encoding peptidylarginine deiminase 6.
2. The polypeptide of claim 1 which is of mammalian origin, preferably of human origin.  
5
- 2.3. The polynucleotide according to claim 2 said polynucleotide encoding SEQ ID NO:1 or SEQ ID NO:3
4. The polynucleotide according to claim 3 said polynucleotide comprising the nucleotides 6-2051 of SEQ ID NO:2 or nucleotides 20-2077 of SEQ ID NO:4  
10
- 3.5. The polynucleotide according to claim 3 said polynucleotide consisting of the nucleotides 6-2051 of SEQ ID NO:2 or nucleotides 20-2077 of SEQ ID NO:4.
- 5.6. A recombinant expression vector comprising the polynucleotide according to claims 1-5.  
15
- 6.7. A polypeptide encoded by the polynucleotide according to claims 1-5 or the expression vector according to claim 6.
- 7.8. A cell transfected with a polynucleotide according to claims 1-5 or the expression vector according to claim 6.  
20
- 8.9. The cell according to claim 8 which is a stable transfected cell which expresses the polypeptide according to claim 7.
- 9.10. A method to produce the polypeptide of claim 7 the method comprising culturing the cells of claim 8 under conditions wherein said protein is produced and recovering said protein from the culture.  
25
11. A method for identifying compounds that affect the enzymatic function of peptidylarginine deiminase 6, the method comprising the steps of
  - a) contacting said peptidylarginine deiminase 6 with an arginine containing substrate
  - b) contacting said mixture with a test compound
  - c) measuring the arginine to citrulline conversion and
  - d) comparing said conversion with peptidylarginine deiminase activity in the absence of a test compound.  
30

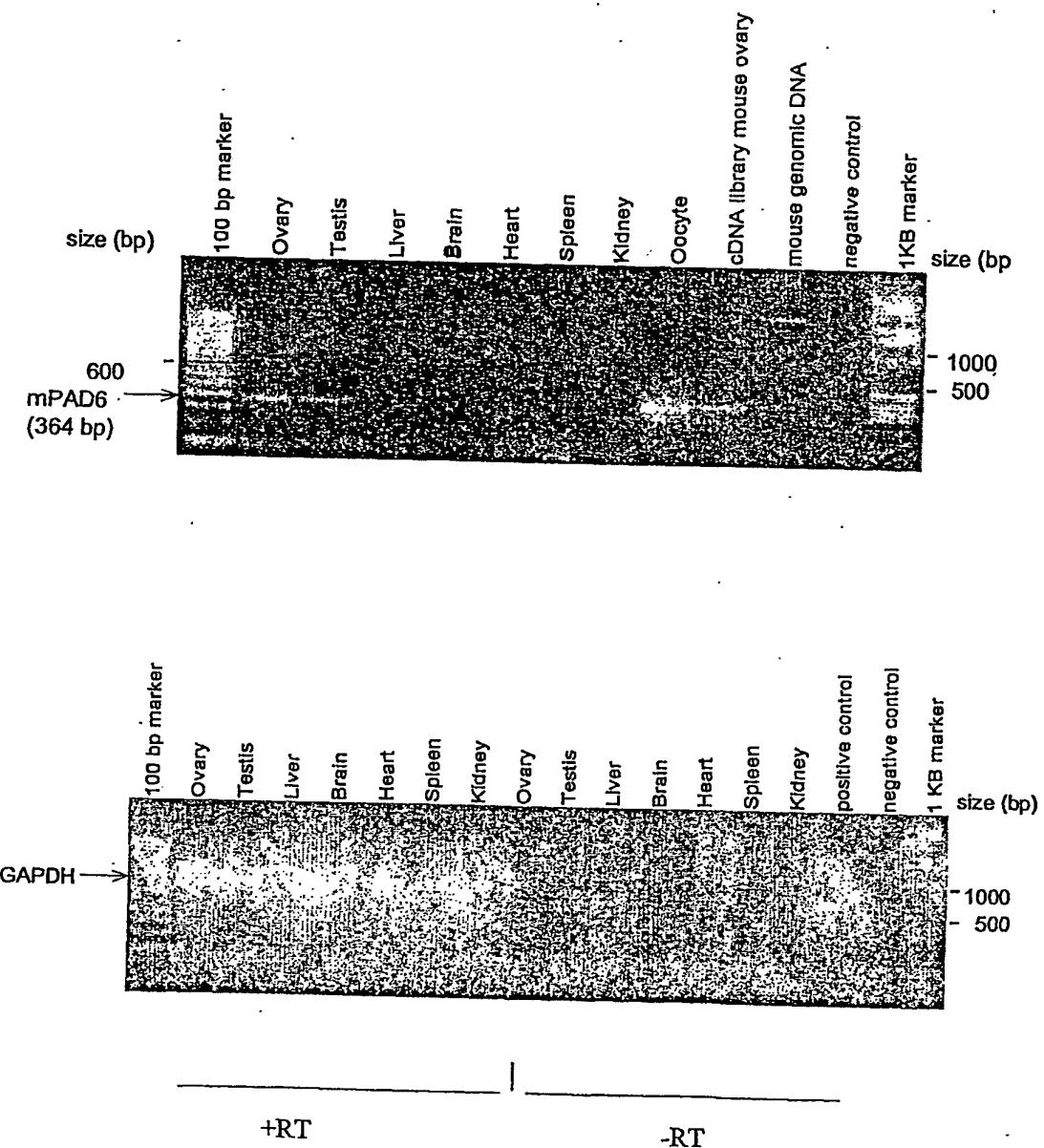


Figure 1

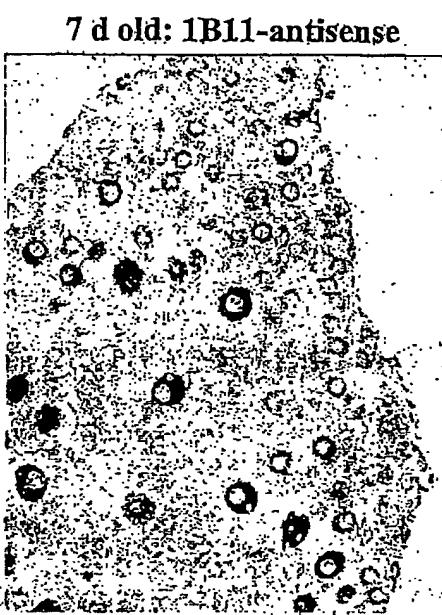
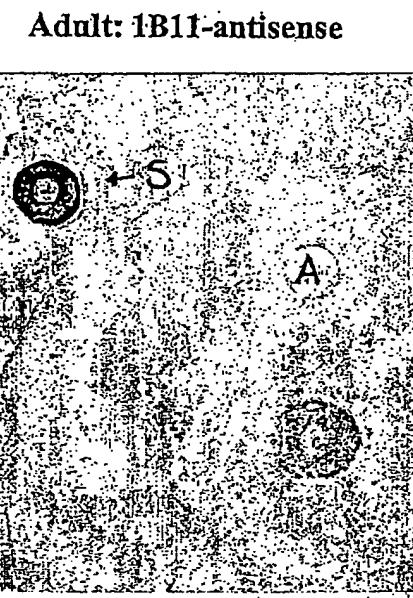


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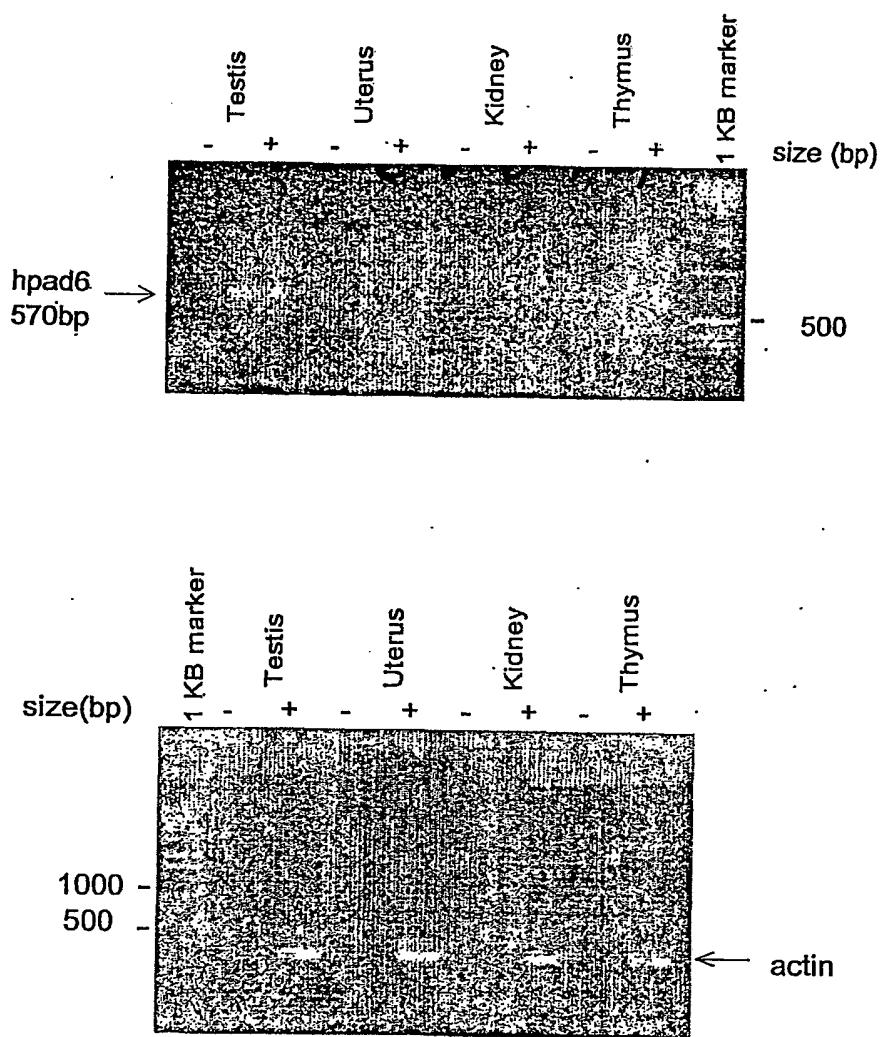


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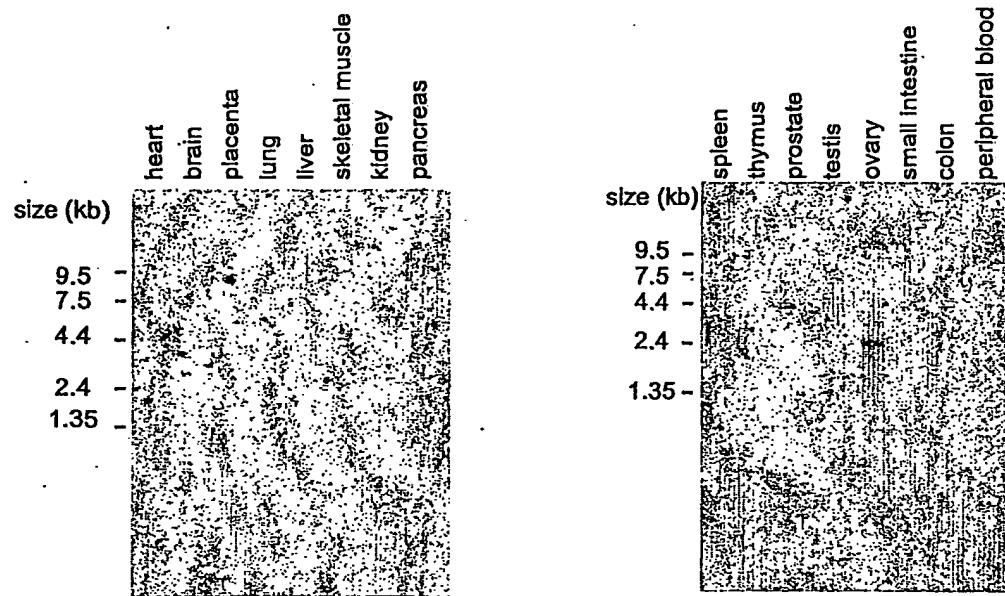


Figure 4

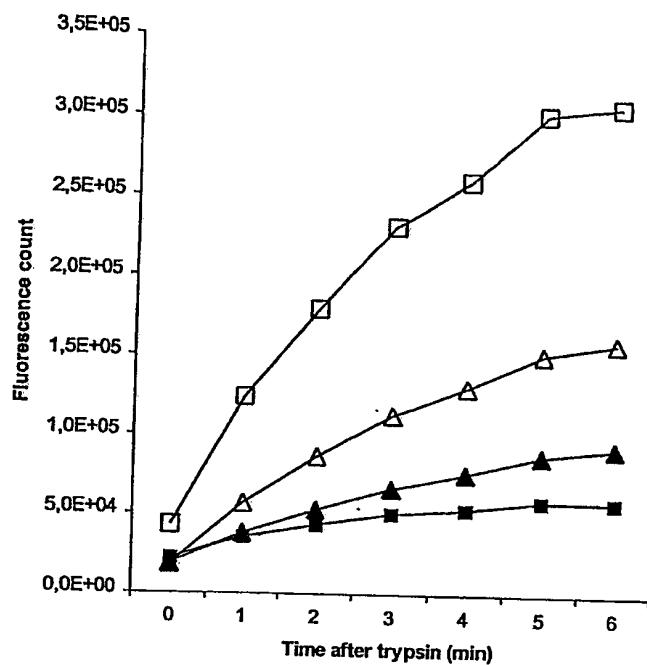


Figure 5

## SEQUENCE LISTING

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